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Synergistic Activation of the Human Immunodeficiency Virus Type 1 Promoter by the Viral Tat Protein and Cellular Transcription Factor Sp1

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We have previously shown that the human immunodeficiency virus type 1 (HIV-1) Tat protein can activate a synthetic promoter containing consensus-binding sites for the cellular transcription factor Sp1. In this report, we show that a GAL-Tat fusion protein targeted via GAL4 DNA-binding sites can also *trans* activate an HIV-1 LTR promoter independently of the *trans*-activation response region. To show that the *trans* activation of the promoter by Tat directly involves the Sp1 protein, we have targeted a GAL-Sp1 fusion protein to the long terminal repeat promoter via upstream GAL4-binding sites. In the presence of Tat and GAL-Sp1, the promoter is synergistically *trans* activated at the transcriptional level, indicating that Tat and Sp1 functionally interact to *trans* activate the HIV-1 promoter. The Sp1 synergism is relatively specific, since another chimeric transcriptional activator, GAL-VP16, does not appear to be significantly synergistic with Tat.

The Tat protein of the human immunodeficiency virus (HIV) is a powerful *trans* activator of viral gene expression from the viral promoter located in the long terminal repeat (LTR) (1, 31). An unusual feature of Tat *trans* activation is that Tat functions through a downstream promoter element or *trans*-activation response element (TAR) (24). The functional TAR element is not the DNA sequence itself but is contained in the secondary structure of the 5′ region of the nascent RNA transcripts (3, 7, 8, 25, 28, 30) whose primary function appears to be to target Tat to the promoter. This targeting role has been demonstrated by replacing TAR with the target sequences of other RNA-binding proteins and *trans* activating the promoter with the cognate Tat fusion protein (29, 33). The mechanism of action of Tat once it is so tethered to the promoter region is poorly understood, but it appears that *trans* activation by Tat can occur at the level of both transcriptional initiation and elongation (5, 15, 22, 30) and perhaps involves HIV promoter sequences (2).

In an effort to understand whether Tat might function in a manner similar to that of other eukaryotic DNA sequence-specific *trans* activators in conjunction with an upstream promoter element(s) and to identify this element, we previously tested the activity of Tat on synthetic promoters (13). In that study, Tat fusion proteins containing the DNA-binding domain of the yeast transcriptional activator GAL4 were targeted to the promoters via upstream DNA sequences for GAL4 binding. *trans* activation of the synthetic promoter was dependent on the presence of DNA-binding sites for the transcription factor Sp1, suggesting that Tat might activate transcription through interaction with the Sp1 protein or another cellular protein that binds to the Sp1 DNA-binding motif. In the present report, we show that the Sp1 protein itself is directly involved in Tat-mediated *trans* activation of the HIV type 1 (HIV-1) LTR.

Activation of the HIV-1 LTR by a GAL4-Tat fusion protein. A synthetic promoter requires only a TATA box and Sp1-binding motifs downstream of the GAL4-binding motif to be *trans* activated by the GAL4-Tat fusion protein. To determine whether the HIV-1 LTR could also be *trans* activated by a GAL4-Tat fusion protein through GAL4-binding motifs, we constructed a plasmid (pG5LTR-CAT) which expresses the bacterial chloramphenicol acetyltransferase (CAT) gene under the transcriptional control of the HIV-1 LTR downstream of five GAL4-binding motifs. pG5LTR-CAT contains HIV-1 LTR sequences between −159 and +77 from pU3R111 (*Ava*I to *Nco*I) (31) inserted between the *Xba*I (T4 DNA polymerase-blunted) and the *Nco*I sites of pG5BCAT (23) (Fig. 1). The GAL-Tat48 fusion protein expressed from pGAL4-Tat48 (13) contains the N-terminal 147-amino-acid DNA-binding domain of the yeast transcriptional *trans* activator GAL4 fused to the N-terminal 48 amino acids of Tat and hence lacks the arginine-rich basic region of Tat which is normally required for TAR targeting of Tat to the HIV promoter. Subconfluent cultures of HeLa cells were transfected with different LTR CAT constructs, along with either pGAL4-Tat48 or a control plasmid, pGAL4 (pSG424) (27), which contains only the GAL4 N-terminal (1 to 147) sequence. As expected, neither pGAL4 nor pGAL4-Tat48 activates CAT expression from the control (pLTR-CAT) (18) construct lacking the GAL4-binding motifs (Fig. 2). However, cotransfection of pGAL4-Tat48 with pG5LTR-CAT results in approximately 18.2-fold activation of CAT expression over that of the pGAL4 control (Fig. 2). This is less (18.2- versus 60-fold) than the level of *trans* activation by pGAL4-Tat48 of a synthetic promoter containing only Sp1-binding sites and a TATA box and suggests that either the LTR has additional negative regulatory elements or the configuration of the Sp1-binding sites and TATA box relative to the positioning of the GAL4 sites affects *trans* activation.

Since the GAL4-binding motif contains a basic region of its own which is required for nuclear localization of the fusion protein, the possibility remained that the GAL-Tat fusion protein was activating CAT expression through low-level nonspecific binding to TAR. Therefore, TAR was inactivated by deleting the sequence between +25 and +38 nucleotides of the transcriptional start site in pG5LTR-CAT (from the *Bgl*II site to the *Sac*I site and blunted with T4 DNA

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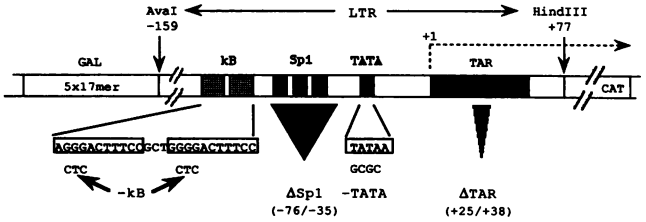

FIG. 1. Structure of pG5LTR-CAT and the locations of LTR mutations. pG5LTR-CAT was derived from pG5BCAT as described in the text. LTR mutations were transferred into pG5LTR-CAT from mutated LTR CAT constructs kindly supplied by G. Nabel (21).

polymerase) (pG5ΔTAR-CAT [Fig. 1]). The presence of an inactive TAR in pG5ΔTAR-CAT reduced *trans* activation by pGAL4-Tat48 only slightly, from 18.2- to 14.1-fold (Fig. 2). This modest reduction in activation with pG5ΔTAR-CAT was not significant. In a repetition of the same experiment, pGAL4-Tat48 produced a 15.9- and a 17.8-fold activation with pG5LTR-CAT and pG5ΔTAR-CAT, respectively (Fig. 2 [legend]). Therefore, *trans* activation of the HIV LTR by the GAL-Tat48 fusion protein is independent of TAR. To determine whether *trans* activation of pG5LTR-CAT was dependent on the presence of the Sp1 sites, all three Sp1 sites were deleted from the LTR in pG5ΔSp-CAT (Fig. 1). Deletion of the Sp1 sites reduced the already low level of basal promoter activity of pG5LTR-CAT still further, and *trans* activation of pG5ΔSp-CAT by pGAL4-Tat48

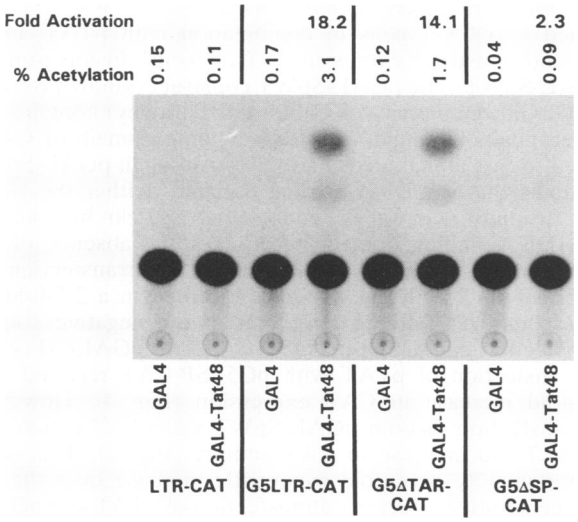

FIG. 2. *trans* activation of different LTR CAT constructs by pGAL4-Tat48. HeLa cells on 100-mm^2 plates were transfected by $CaPO_4$-DNA coprecipitation with 2 μg of the indicated CAT or activator plasmid DNA. The level of CAT activity of the cell extracts was determined 48 h posttransfection (10). Fold *trans* activation is expressed as the percent acetylation with each LTR CAT construct with pGAL-Tat48 divided by the percent acetylation in cultures cotransfected with the negative control plasmid pGAL4 (pSG424) (27). All of the transfection assays were repeated at least twice, and fold *trans* activations in CAT assays from repeat experiments are included in the legends for Fig. 2 through 5 to indicate the reproducibility of the results. In a second experiment, pGAL4-Tat48 *trans* activated pLTR-CAT 0-fold, pG5LTR-CAT 15.9-fold, pG5ΔTAR-CAT 17.8-fold, and pG5ΔSP-CAT 3.4-fold.

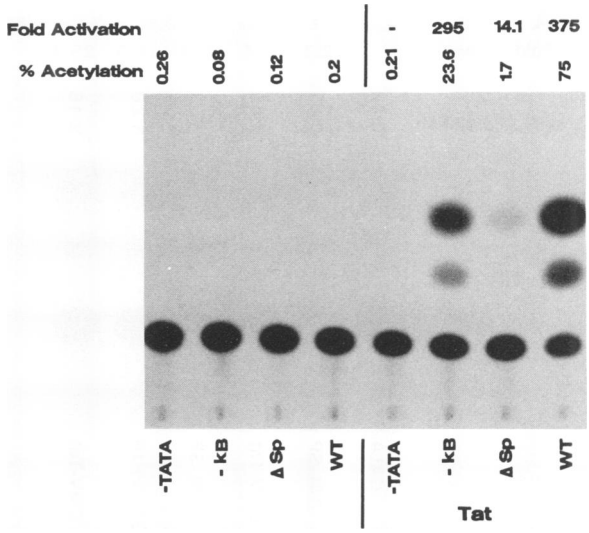

FIG. 3. *trans* activation of mutated LTR CAT constructs by pTAT. Each of the G5LTR-CAT mutant constructs described in the legend to Fig. 1 was cotransfected with or without pTAT. CAT assays were carried out as described in the legend to Fig. 2. In a second experiment, Tat *trans* activated mutant TATA (−TATA) 2.5-fold, mutant κB (−κB) 317-fold, ΔSP 26.4-fold, and wild type (WT) 508-fold.

was reduced from 18.2- to 2.3-fold. The low level of activation in the absence of the Sp1 sites was reproducible and was found to be in the two- to fourfold range; however, in absolute terms, this activation increased promoter activity only to the basal activity level of pG5LTR-CAT.

Effect of specific promoter mutations on *trans* activation by Tat. The three major defined elements of the HIV-1 promoter are the enhancer, Sp1-binding sites, and the TATA box. Figure 1 shows the location of the LTR enhancer

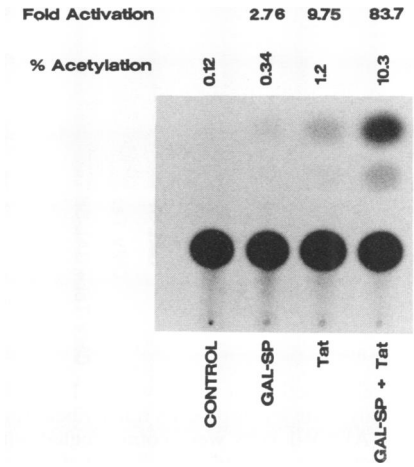

FIG. 4. Synergistic activation of pG5ΔSP-CAT by Tat and Sp1. CV-1 cells were transfected as described in the legend to Fig. 2 with pG5ΔSP-CAT and either control DNA (pGAL4) or pTAT and pGAL-SpWT DNA, as indicated. CAT assays were carried out as described in the legend to Fig. 2. In a second experiment, pGALΔSP-CAT was *trans* activated 4.1-fold by pGAL-SpWT, 8.7-fold by pTAT, and 69.3-fold by cotransfection of pTAT and pGAL-SpWT.

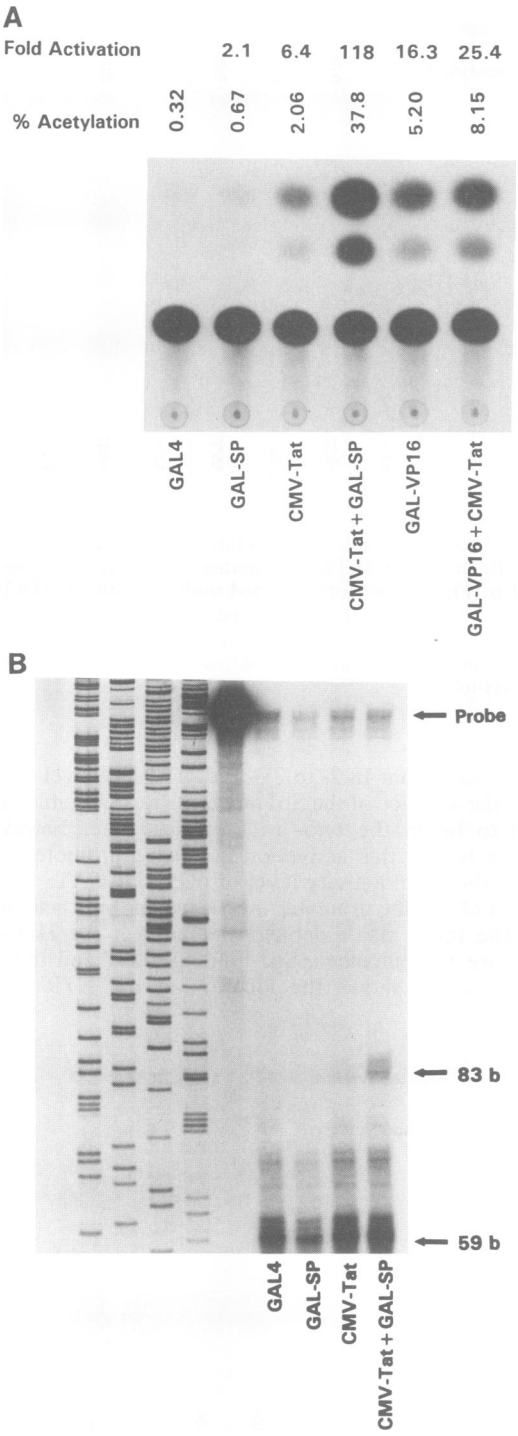

FIG. 5. Synergistic activation of Tat with Sp1 is preferential and increases the steady-state levels of RNA transcribed from the HIV-1 LTR promoter. (A) CV-1 cells were cotransfected with pG5ΔSP-CAT and each activator plasmid as indicated. CAT assays were carried out as described in the legend to Fig. 2. In a second transfection experiment, pG5ΔSp-CAT was *trans* activated 3.4-fold by pGAL-SpWT, 14.8-fold by pCMV-TAT, 194-fold by pCMV-TAT and pGAL-SpWT, 30.2-fold by pGAL4-VP16, and 59.7-fold by pCMV-TAT and pGAL4-VP16. (B) Total cellular RNA was isolated from duplicates of the transfection plates that were assayed for CAT activity in panel A and hybridized to a SP6 polymerase ^{32}P-riboprobe transcribed from pGEM23 (kindly provided by M. Laspia) linearized with *Xba*I. Hybridization and S1 nuclease digestion conditions were carried out as previously described (14). S1 nuclease digestions were analyzed on an 8% polyacrylamide–7 M urea sequencing gel, along with a reference sequence ladder for size determinations of the protected fragments and a lane which contained only the labeled probe.

containing the two NF-κB-binding sites, the three Sp1-binding motifs, and the TATA box. To generate mutants in each of these promoter elements, we transferred a series of promoter mutations previously described by Nabel et al. (21) into pG5LTR-CAT (Fig. 1). With the κB mutation, the GGG triplet in each of the binding sites is replaced by a CTC triplet (pG5ΔKB-CAT). This alteration in the κB-binding motif prevents NF-κB binding. The Sp1 mutation (pG5ΔSP-CAT) deletes all three Sp1-binding motifs as well as the 10 adjacent nucleotides downstream of the Sp1 sites. The TATA box mutation substitutes GCGC for TATA in the HIV TATA box (pG5ΔTATA-CAT). Each of these LTR CAT constructs was cotransfected with pTAT, which expresses Tat under the control of the simian virus 40 early promoter (22). The parental wild-type pG5LTR-CAT plasmid was *trans* activated 375-fold by cotransfection by pTAT (Fig. 3). Deletion of the TATA box resulted in a complete loss of Tat responsiveness. Inactivation of the κB DNA-binding motif (pG5ΔKB-CAT) resulted in a modest 21% decrease in Tat *trans* activation (295- versus 375-fold *trans* activation) (Fig. 3). However, the Sp1 deletion (pG5ΔSP-CAT) resulted in a more pronounced reduction in *trans* activation by pTAT, which was only 3% of the level of *trans* activation of pG5LTR-CAT (14- versus 375-fold *trans* activation, respectively) (Fig. 3). The effects of these mutations are in agreement with previous studies, which have indicated that the TATA box is required for Tat *trans* activation and that the deletion of all three of the Sp1 sites results in a very drastic reduction in the level of *trans* activation by Tat, whereas deletion of the κB motif results in a much less dramatic effect on Tat *trans* activation (11, 20, 21, 24).

Synergistic activation of the HIV-1 promoter by Tat and transcription factor Sp1. To determine whether Tat and Sp1 can function synergistically, pG5ΔSP-CAT was cotransfected into CV-1 cells in combination with pTAT and a plasmid construct expressing a GAL4-Sp1 fusion protein, pGAL-SpWT. The pGAL-SpWT construct (kindly provided by G. Gill, University of California, Berkeley) contains the N-terminal 147-amino-acid DNA-binding domain of GAL4 fused to Sp1 amino acids 83 to 778. Although pGAL-SpWT contains the Sp1 DNA-binding domain, neither the GAL-Sp1 fusion protein nor the endogenous Sp1 can bind via the Sp1 DNA-binding domain because of the absence of the Sp1-binding motif in the promoter. Cotransfection of pG5ΔSP-CAT with pGAL-SpWT resulted in a 2.7-fold increase in CAT activity over that of the negative control transfection containing pG5ΔSP-CAT and pGAL4 (Fig. 4). Cotransfection of pTAT with pG5ΔSP-CAT resulted in a 9.7-fold increase in CAT expression (Fig. 4). However, cotransfection of both pGAL-SpWT and pTAT resulted in an 83.7-fold increase in CAT activity (Fig. 4). This is an approximately sevenfold synergistic increase over the expected additive effect of cotransfection of pTAT and pGAL-SpWT with pG5ΔSP-CAT. These fold *trans*-activation values are in agreement with those from repeated transfection experiments. For example, a second transfection gave the following *trans*-activation values: GAL-Sp1, 4.1-fold; Tat,

8.7-fold; and GAL-Sp1 and Tat combined, 69.3-fold, for a 5.5-fold synergistic effect.

The degree of apparent synergism displayed between Tat and the GAL-Sp1 fusion protein on pG5ΔSP-CAT does not completely compensate for the decrease in the fold *trans* activation displayed by Tat on the LTR promoter lacking the Sp1-binding motifs (Fig. 3). The level of synergy should be four times higher in the experiment in Fig. 4 to compensate for the decrease from a 375-fold *trans* activation of the wild-type pG5LTR-CAT by Tat to the 14-fold *trans* activation of pG5ΔSP-CAT by Tat (Fig. 3). This difference is probably due to the lower activity of the GAL-Sp1 fusion protein compared with that of native Sp1 functioning through the Sp1-binding motif. This appears to be the case with the GAL-Tat fusion protein compared with the native Tat protein. The GAL-Tat58 fusion protein, which contains the arginine-rich basic region, can *trans* activate the wild-type pLTR-CAT via TAR but is substantially less active than the native Tat protein is on pLTR-CAT (12, 13). Alternatively, additional cellular activator proteins may bind to DNA motifs that overlap with the Sp1-binding motifs which have been deleted.

The possibility remained that pGAL-SpWT was activating CAT expression by stimulating Tat synthesis through activation of the Sp1-dependent simian virus 40 early promoter of pTAT and not through a synergistic interaction with the Tat protein. Accordingly, pCMV-TAT, which expresses Tat from the cytomegalovirus (CMV) immediate-early promoter, was cotransfected with pGAL-SpWT. pCMV-TAT was constructed from pgTAT (17) (kindly supplied by B. Cullen) by inserting the *Blg*II (blunted)-to-*Sal*I Tat sequences from pTAT into the *Sal*I (blunted) and *Xho*I sites of pgTAT. In this experiment, pGAL-SpWT transfected alone with pG5ΔSP-CAT activated CAT expression 2.1-fold and pCMV-TAT alone activated CAT expression 6.4-fold (Fig. 5A). Cotransfection of pCMV-TAT with pGAL-SpWT *trans* activated pG5ΔSP-CAT 118-fold (Fig. 5A). This represents a 13-fold synergistic effect. These fold *trans*-activation values were consistent and resulted in a somewhat greater synergistic effect than was found with the pTAT construct. These results indicate that the Tat protein and transcription factor Sp1 can functionally interact in the *trans* activation of the HIV-1 promoter.

To determine whether the synergism between Tat and Sp1 results in elevated levels of transcription from the LTR promoter, S1 nuclease protection assays were performed. Total cellular RNA was isolated from duplicates of transfection plates that were assayed for CAT activity (Fig. 5A) and hybridized to a ^{32}P-riboprobe transcribed by SP6 polymerase from pGEM23 (kindly provided by M. Laspia) linearized with *Xba*I. pGEM23 contains the HIV-1 sequences from −117 to +83 of the transcriptional start site. SP6 polymerase generates a 200-base RNA probe from the linearized template. RNA correctly initiated from the HIV-1 promoter should protect radiolabeled probe fragments of two sizes, one of 59 bases representing prematurely terminated nascent RNAs whose levels are unaffected by Tat and a second species of 83 bases representing read-through transcription to full-length mRNA which is increased by Tat (15). As expected, RNAs from cells transfected with pGAL-SpWT, pCMV-TAT, or pGAL-SpWT and pCMV-TAT combined did not show an increase in the amount of the 59-base fragment protected; however, cotransfection of pCMV-TAT and pGAL-SpWT did increase the amount of the 83-base fragment protected, indicating that synergism of Tat and Sp1 increases the steady-state level of mRNA correctly initiated from the HIV-1 transcriptional start site.

In an effort to determine whether the Sp1 synergism displayed by Tat is preferential or can be generalized to other *trans* activators, a second construct, pGAL4-VP16 (26) (kindly provided by I. Sadowski), was cotransfected with pCMV-TAT. pGAL4-VP16 encodes a GAL4 chimeric protein containing the 147 N-terminal amino acids of GAL4 fused to the 78 C-terminal amino acids of the herpes simplex virus protein VP16. Unlike Sp1, VP16 contains a highly acidic activation domain that is believed to function through a direct interaction with TFIID or TFIIB (16). Cotransfection of pGAL4-VP16 with pG5ΔSP-CAT produced a 16.3-fold *trans* activation, cotransfection of pCMV-TAT with pG5ΔSP-CAT alone produced a 6.4-fold *trans* activation, and cotransfection of pGAL4-VP16 in combination with pCMV-TAT produced only a 25.4-fold *trans* activation on pG5ΔSP-CAT (Fig. 5A). In a second experiment, pG5ΔSP-CAT was activated 14.8-fold by pCMV-TAT, 30.2-fold by pGAL4-VP16, and 59.7-fold by a combination of pCMV-TAT and pGAL4-VP16; therefore, less than 2-fold synergism between Tat and pGAL4-VP16 is displayed.

The nature of the functional interaction between Tat and Sp1 is not yet understood. We have been unable to demonstrate a specific physical interaction between purified Sp1 and purified Tat protein in vitro by either chemical crosslinking and coimmunoprecipitation or by the formation of a supershift complex of purified Sp1 and Sp1-specific oligonucleotide in the presence of purified Tat protein in gel retardation assays. Additional cellular proteins might be required for the formation of a functional Sp1-Tat complex. This is supported indirectly by our cotransfection experiments with *Drosophila* cells in culture (12). Tat has been shown to be inactive in *Drosophila* cells, which do not contain Sp1 protein (4, 19). Cotransfection of an active Sp1 expression vector and pTAT with pLTR-CAT does not potentiate Tat *trans* activation, indicating that either a Tat-specific inhibitor is in *Drosophila* cells or an additional human cellular protein is required for Tat-Sp1 *trans* activation (12). Whether any of the recently described cellular proteins which appear to increase Tat *trans* activation in vivo (6, 9) or in vitro (18, 34) are part of an Sp1-Tat activation pathway awaits further investigation.

After this report was submitted, Southgate and Green (32) reported similar results using a GAL4 fusion protein to target Tat upstream of the HIV-1 promoter and also concluded that Tat could cooperate with promoter-bound Sp1.

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